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HIV Drug Resistance in Sub-Saharan Africa – Implications for Testing and Treatment

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1. Introduction

This chapter will review the current evidence surrounding the emergence of HIV drug resistance in sub-Saharan Africa, the current guidelines for the drug resistance testing, and their implication. Data from a cohort study in the Free State province of South Africa will be used as an applied example in the discussion.

2. ARV and mechanism of drug resistance emergence

The first antiretroviral therapy (ARV) drug, azidothymidine (zidovudine, originally developed to treat cancer), was discovered to inhibit the reverse transcriptase (RT) enzyme of HIV in 1986 (Yarchoan, et al. 1986). Since then, more inhibitor classes to other essential steps of viral replication have been discovered (Barbaro, et al. 2005). The ability of HIV to mutate and recombine frequently allows it to evade individual ARV rapidly (Aboulker and Swart 1993). In 1996, the introduction of combination ARV, often termed highly active antiretroviral therapy (HAART), introduced a dramatic treatment response to patients. HAART delays disease progression, reduces AIDS mortality (by up to 70% annually) and partially restores CD4 T cells, but cannot clear HIV infection (Palella, et al. 1998). Furthermore, HAART delays the emergence of resistant HIV isolates, which accumulated rapidly during the pre-HAART era, when mono-, or dual-, therapy were in use (Kuritzkes 2007).

Under multiple ARV drug selection (usually 3 agents, balancing clinical efficacy and patient tolerability), HIV is successfully suppressed (Larder, et al. 1995). However, given time or opportunity (such as when therapy is interrupted or not adhered to), HIV can acquire resistance to one or more agents in HAART regimens, requiring changing of failed ARV components. Drug toxicity is another major health concern (Carvajal-Rodriguez, et al. 2007, Larder, et al. 1993, Larder, et al. 1995). Although some drug escape mutants are impaired in replication fitness, others seem unaffected or may even have increased fitness (Armstrong, et al. 2009, Garcia-Lerma, et al. 2004, Prado, et al. 2002). Similar to escape seen in natural selection, reversion to wild type virus occurs rapidly following treatment cessation. Drug

compensatory mutations also arise secondarily to restore fitness in the mutants harbouring costly escape (Gandhi, et al. 2003, Stanford University 2011).

Furthermore, intra-subtype and inter-subtype differences in HIV genetic sequences evoke different pathways leading to escape (Pieniazek, et al. 2000). Therefore, concerted efforts, surveillance programs and comprehensive databases have been set up to describe subtype-specific drug resistance mutations and their accessory mutations (which may either have predisposing, compensating or augmenting effects to drug concurrent resistance mutations) (Bennett, et al. 2009, Gifford, et al. 2007, Stanford University 2011).

2.1 HIV, HAART and drug resistance in sub-Saharan Africa

In sub-Saharan Africa, 22.5 million people (5% of adult population) are infected with HIV, effectively harbouring over two thirds of the world prevalence (33.3 million) (UNAIDS/WHO 2011). Furthermore, in 2009, HIV infected over 1.8 million adults and children in the sub-Saharan region, and caused 1.3 million deaths through AIDS. This represents public health emergency, requiring immediate effective intervention. However, resource limitation, escalating disease burden and established stigmata remain major challenges to effective intervention. In particular, poverty and resource limitations undermine HIV control by constraining the access, availability and affordability of HIV testing, anti-retroviral therapy (ARV), follow up, and skilled staff (UNAIDS/WHO 2011).

Similarly, sub-Saharan African nations lacked the resources to research and develop their own ARV and HAART. Fortunately, although the ARV and HAART regimens were originally designed to inhibit subtype B HIV-1, they are also efficacious against other subtypes, including C - the dominant subtype infecting sub-Saharan Africa (Frater, et al. 2001, Gordon, et al. 2003, Kantor, et al. 2005). In the *pol* gene subtype C varies from subtype B by 10-12% at the nucleotide level, yet these genotypic differences do not appear to confer major pre-therapy drug resistance, although may be associated with more accessory resistance mutations and different molecular pathways to resistance (Frater, et al. 2002, Gordon, et al. 2003, Pieniazek, et al. 2000, Robertson, et al. 2000, Sanches, et al. 2007, Velazquez-Campoy, et al. 2001).

As HAART became available, many sub-Saharan Africa states initiated large scale primary care dispensing of HAART to qualifying patients according to the CDC and WHO guidelines (Department of Health 2003). However, the regional circulating strains of HIV remain poorly characterised in their molecular epidemiology and genotypic profiles. In addition, various mono- and dual-ARV are also indicated in the prevention of mother-to-child transmission (PMTCT) program of different sub-Saharan regions, exposing patients to potential resistance development (Jourdain, et al. 2004). Therefore, ahead of the mass dispensing of HAART, it is important to study the molecular characterisation and baseline pre-therapy drug resistance profiles of subtype C HIV-1 in South Africa (Department of Health 2003, Gilks, et al. 2006). This will also contribute to the long-term follow up of resistance development.

2.2 Characterising drug resistance

There are three types of ARV resistance (DHHS-Panel 2011):

- Clinical resistance: occurs when viral replication continues despite HAART institution, and carries direct clinical impact. Clinical criteria exist to define virological (increase

- viral load, VL) and immunological failure (poor increase or decreasing of CD4 cell count and development of opportunistic infection) during HAART.
- Phenotypic resistance: can assess viral growth in the presence of ARV *in vitro*. This test is highly specialized (requiring recombination of HIV *pol* gene with a laboratory viral backbone) and is time consuming (viral culture and measurements take three to four weeks). The test is more expensive, and has no proven advantage over genotypic resistance testing, and may suffer reduced sensitivity in the detection of minority resistant mutant isolates (when the mutants are present at less than 20% of circulating viral population).
 - Genotypic resistance: is conducted to sequence the *RT* and *protease* genes for the detection of known resistance mutations (*Integrase* sequencing can also be done, on request). The test is considerably less expensive, time consuming and more widely available when compared to the phenotypic resistance assay (although the level of expertise required is still very high and not common to routine diagnostic laboratories). Furthermore, different sequencing assay techniques could be employed to improve sensitivity of detecting minority resistant mutants. A 'virtual phenotype' can also be imputed to measure the phenotypic impact of a mutation using laboratory strain manipulation. To date, global databases including Stanford HIV drug resistance database (Stanford HIV db) have collated, analysed and summarised considerable amounts of different genotype-treatment, genotype-clinical, and genotype-phenotype evidence based academic publications.

The identification of drug resistance in sub-Saharan Africa has become a major issue in patients receiving HAART and in drug-naïve individuals (Shekelle, et al. 2007). Guidelines exist for the surveillance of both - the World Health Organisation (WHO) and Stanford HIV db publish a list of mutations for use in surveillance of transmitted drug resistance (TDRM) and the International AIDS Society (IAS) produce a list for use in the monitoring of treated populations (HRDS-Team 2003, Johnson, et al. 2008, Stanford University 2011). The WHO, Stanford HIV db and IAS committees regularly update the definitions and clinical significance (phenotypic resistance, clinical association and outcome meta-analyses) of drug resistance mutations and drug associated polymorphisms (Bennett, et al. 2009, Shafer, et al. 2007). However, authorities disagree on the classification of naturally occurring non-clade B polymorphisms, drug-associated polymorphisms not associated with direct measurable drug resistances, and polymorphisms associated with reversion of previous drug-resistance mutation (Stanford University 2011). Therefore, many of the Stanford db's 'potentially low grade' and 'low grade' resistance mutations are excluded from the IAS and WHO surveillance lists, showing there are discrepancies between the key opinion leaders. Recently, the updated TDRM list from the Stanford HIV db has become unified with the WHO guidelines (Gilks, et al. 2006, HRDS-Team 2003). The mutations in the TDRM surveillance list are selected on the basis that they are non-polymorphic, clinically significant and applicable across all subtypes. The implementation of such a standard should facilitate a specific method to assess changes in drug resistance prevalence in populations over time (Garcia-Diaz, et al. 2008, HRDS-Team 2003, Seebregts, et al. 5-8 June 2007). It is clearly ideal to have a single mutations list to screen all populations, however the possibility exists that for South Africa where the dynamics of therapy provision and the more recent introduction of universal access to HAART makes for a unique situation, a subtype C-specific list may become more applicable (SATuRN-Database, et al. 2011).

3. Molecular epidemiology and genotypic drug resistance in treatment-naïve HIV population of South Africa

In South Africa (SA, Figure 1), 5.5 million people are infected with HIV (ASSA 2007, UNAIDS/WHO 2011, Venter 2005). Since 2004, the SA government has made antiretroviral therapy (ARV) available to HIV positive individuals through public sector ARV programs - an initiative with significant clinical, public health and economic impact (Department of Health 2003, Egger, et al. 1997, Frater 2002, Palella, et al. 1998, Sow, et al. 2007, Venter 2005).

Until recently, only a few small to medium-sized South African cohorts have reported the molecular characterisation and baseline pre-therapy resistance profiles of HIV infection, predominantly amongst the KwaZulu Natal, Gauteng and Limpopo provinces (Bessong, et al. 2006, Gordon, et al. 2003, Pillay, et al. 2002, Shekelle, et al. 2007). There are also emerging data on drug resistance from other major cities of sub-Saharan countries such as Uganda, Tanzania, Botswana, Zimbabwe, Zambia, Malawi and Kenya (Bussmann, et al. 2005, Eshleman, et al. 2009, Gordon, et al. 2003, Hamers, et al. 2010, Kamoto and Aberle-Grasse 2008, Lihana, et al. 2009, Mosha, et al. 2011, SATuRN-Database, et al. 2011, Shekelle, et al. 2007, Tshabalala, et al. 2011). There is currently no similar information available from other less urbanised regions of South Africa, such as Free State Province (Figure 1) with a population of approximately 3 million, an antenatal clinic HIV prevalence of 33.9%, and the life expectancy estimate of 46.5 years (ASSA 2007). Under the Free State Comprehensive Care, Management and Treatment of HIV and AIDS program, highly active antiretroviral therapy (HAART) has been available since 2004 as a combination stavudine and lamivudine with either nevirapine or Kaletra, and has significantly reduced the number of HIV-related deaths (hazard ratio 0.14) (Fairall, et al. 2008).

In patients who report that they are drug-naïve, resistance mutations might either reveal transmitted variants or might indicate that patients are not, in fact, drug-naïve or are unaware of previous ARV exposure (Garcia-Diaz, et al. 2008). The extent to which unrecognized access contributes to the level of resistance in patients enrolling onto public sector ARV is unknown in South Africa, and very difficult to measure. Prior to the introduction of the public sector ARV treatment programme, ARV drugs were available for the PMTCT through private clinics and other unregulated routes, although previous studies suggest a low prevalence of baseline drug resistance in South Africa (Bessong, et al. 2006, Gordon, et al. 2003, Pillay, et al. 2002, Shekelle, et al. 2007).

Here, the discussion on molecular epidemiology and pre-therapy drug resistance will be applied to a South African clinical cohort study (Huang, et al. 2009). In the Free State province of South Africa, 425 HIV type-1 (HIV-1)-positive patients newly recruited to the public sector ARV treatment programme and reporting to be drug-naïve were studied. The molecular epidemiology of HIV-1 infection within this region were characterised, and the prevalence of drug resistance and other polymorphisms associated with drug exposure were measured. A correlation existed between low CD4 T cell counts and drug-selected polymorphisms, suggesting that many mutations in drug-naïve individuals are not transmitted, but are the result of acquired resistance through unrecognised ARV access.

The aims of this study were: to describe the viral molecular epidemiology of Free State Province, South Africa (Figure 1), to investigate the prevalence of drug-resistance associated mutations, and resistance to HAART in the pre-therapy cohort and to model the impact of ART availability on baseline antiretroviral resistance.



Left panel: South Africa (in red) at the south of Sub-Saharan Africa (boxed and enlarged from the world map in left lower corner).

Right panel: Free State province (in blue, capital: Bloemfontein) and KwaZulu Natal province (in purple, capital: Durban) are two of the nine provinces in central east region of South Africa.

Fig. 1. Map of South Africa and its provinces, figure modified from original (Wikimedia-Commons 2010)

4. Experimental approach to the Free State cohort

In total, 884 adult patients were recruited at their first visit to government antiretroviral therapy (ART) clinics in the Free State province of South Africa between February and September 2006. Informed consent for additional sampling of plasma for viral sequencing was obtained. All patients had been diagnosed with HIV infection at local primary care clinics, and then referred to district or regional HIV clinics to be assessed for suitability for HAART. On attending the clinic, patients were counseled by trained HIV nurses and asked directly whether they had previously received PMTCT, mono-, dual- or triple antiretroviral therapy. Patients who admitted previous drug exposure were excluded from this analysis. Demographic details, routine CD4 T cell counts and an additional 10ml of peripheral venous blood were collected from each individual patient. The ethics and study design were approved by the regional university and department of health.

The 884 patients were stratified according to their CD4 cell count into 5 groups as follows: <100 cells/ μ l ($n = 195$, 22.1%); 100-199 cells/ μ l ($n = 212$, 24%); 200-349 cells/ μ l ($n = 244$, 27.6%); 350-499 cells/ μ l ($n = 123$, 13.9%) and >500 cells/ μ l ($n = 110$, 12.4%) (Table 1). From this cohort of 884, viral sequences were analysed from 425 patients to form the 'low' (<100 CD4 cells/ μ l; $n = 195$), 'intermediate' (200-349 CD4 cells/ μ l; $n = 120$) and 'high' (>500 CD4 cells/ μ l; $n = 110$) CD4 cell count stratification groups.

For sequencing, RNA was extracted from plasma, converted to cDNA and then amplified by polymerase chain reaction before sequencing using ABI Big Dye terminator kits (Applied Biosystems). The primers used for the PCR and sequencing are described elsewhere (Frater, et al. 2007). From the 425 patients, complete *protease* sequences (99 amino acids) and amino acids 1-530 of *reverse transcriptase* (RT) were successfully. The *pol* gene sequences were submitted to the BioAfrica database and REGA HIV-1 subtype tool for subtype (www.bioafrica.net/subtypetool/html/) and locus identification (de Oliveira and Cassol

2011, de Oliveira, et al. 2005). Phylogenetic analysis and maximum likelihood trees were constructed using the General Time Reversible substitution model with optimised proportions of invariable sites and gamma distribution (GTR+G+I) using PAUP* (version 4.0 beta) and PhyML (version 2.4.4) software. The *pol* sequences of the cohort were compared to 986 well-characterised isolates from the BioAfrica database and those available on the Los Alamos database (www.hiv.lanl.gov) to determine subtype and lineage relationships (Guindon and Gascuel 2003, Los Alamos National Laboratory 2011). The Slatkin and Maddison test was used to assess clustering between the Free State Province sequences and other South African sequences, using MacClade (version 4) (Gifford, et al. 2007).

The *protease* and *RT* sequences were submitted to the Stanford drug resistance database (version 4.3.1, <http://hivdb.stanford.edu/pages/algs/HIVdb.html>) to identify subtype-associated polymorphisms and drug resistance mutations (Bennett, et al. 2008). The Stanford mutation scoring system (Table 1) was used to distinguish accessory mutations (mutation score 0-9) from clinically significant mutations ('potentially low level' resistance (score 10-14), 'low level' (score 15-30), 'intermediate level' (score 30-59) and 'high level' (score ≥ 60) resistance) (Bennett, et al. 2008, Stanford University 2011).

An ordinary differential equation model was devised for this study to describe the dynamics of resistance to a single drug, such as Nevirapine, with a simple genetic resistance profile. The model encapsulates four main processes: selection of drug resistance in hosts treated in the public sector ARV treatment program, selection of drug resistance prior to 'official' treatment by other means such as private prescriptions, transmission of drug resistant strains to new hosts, and reversion to drug-sensitive strains in untreated hosts. Fisher's exact test (two tailed) and chi-square test were used to compare pre-therapy resistance findings between groups with different CD4 cell counts.

5. Molecular epidemiology and drug resistance prevalence in the Free State

In the analysis, there were three key findings. Firstly, there was evidence of multiple introductions of HIV-1 into the Free State, but random distribution of drug resistance-associated polymorphisms. Secondly, the overall prevalence of pre-therapy drug resistance was low, but drug-selected polymorphisms were concentrated among patients with low CD4 T cell counts. Thirdly, mathematical modelling suggested that baseline drug resistance may be driven by exposure to ARVs available through non-governmental routes and that, unless drug availability is controlled, resistance prevalence is likely to rise. In the section below, each of the findings will be described and discussed in more details.

5.1 Characterisation of the Free State province cohort in South African

Table 1 summarises the cohort demography and division for sub-studies. Patients attending antiretroviral clinics in Free State province during 2006 demonstrated a mean CD4 T cell count of 271 cells/ μ l, with 44.8% of patients possessing a CD4 cell counts of less than 200 cells/ μ l, significantly lower than other published chronic HIV cohorts (Kiepiela, et al. 2004). In an earlier study of 2777 chronically HIV infected individuals in the Pelonomi Hospital, Bloemfontein recruited between 1991 and 1997 (van der Ryst, et al. 1998), the mean CD4 T cell counts was 421 cells/ μ l. The low mean CD4 T cell counts seen in the Free State Cohort is likely

Category	Complete cohort	CD4 ⁺ T-cell count					P-value (χ^2 test for trend)
		<100 cells/ μ l	100–199 cells/ μ l	200–349 cells/ μ l	350–499 cells/ μ l	\geq 500 cells/ μ l	
Patients in cohort, <i>n</i> (%)	884	195 (22.1)	212 (24.0)	244 (27.6)	123 (13.9)	110 (12.4)	–
Patients sampled, <i>n</i>	425	195	–	120	–	110	–
Patients with both protease and RT gene sequences, <i>n</i>	390	192	–	112	–	86	–
Mean age, years (\pm SD)	36.1 (9.1)	38 (8.9)	–	35.4 (9.0)	–	34.2 (9.2)	–
Mean CD4 ⁺ T-cell count, cells/ μ l (\pm SD)	271.3 (200.82)	45.36 (28.66)	–	269.96 (40.86)	–	651.80 (178.91)	–
Patients with drug resistance mutations, <i>n</i> (%) ^a	9 (2.3)	7 (3.6)	–	1 (0.9)	–	1 (1.2)	0.134 (0.055)
Total drug resistance mutations, <i>n</i>	11	9	–	1	–	1	–
Patients with non-accessory drug-associated mutations, <i>n</i> (%) ^b	16 (4.1)	13 (6.8)	–	2 (1.8)	–	1 (1.2)	0.015 (0.004)
Total non-accessory drug-associated mutations, <i>n</i>	19	16	–	2	–	1	–
Patients with any polymorphism at drug resistance-associated sites, <i>n</i> (%) ^b	64 (16.4)	37 (19.3)	–	15 (13.4)	–	12 (14.0)	0.193 (0.099)
Total naturally occurring drug-associated mutations, <i>n</i>	73	44	–	15	–	–	–

Table 1. Details of HIV-1 positive patients recruited to three subgroups according to CD4 T cell count

a result of the cohort studied – ARV clinics tend to be enriched with patients presented during symptomatic phase of late HIV. There was no public ARV program available during 1990s. The availability of HAART from the government is expected to improve quality of life and reverse this population-wide fall in CD4 cell counts (Venter 2005).

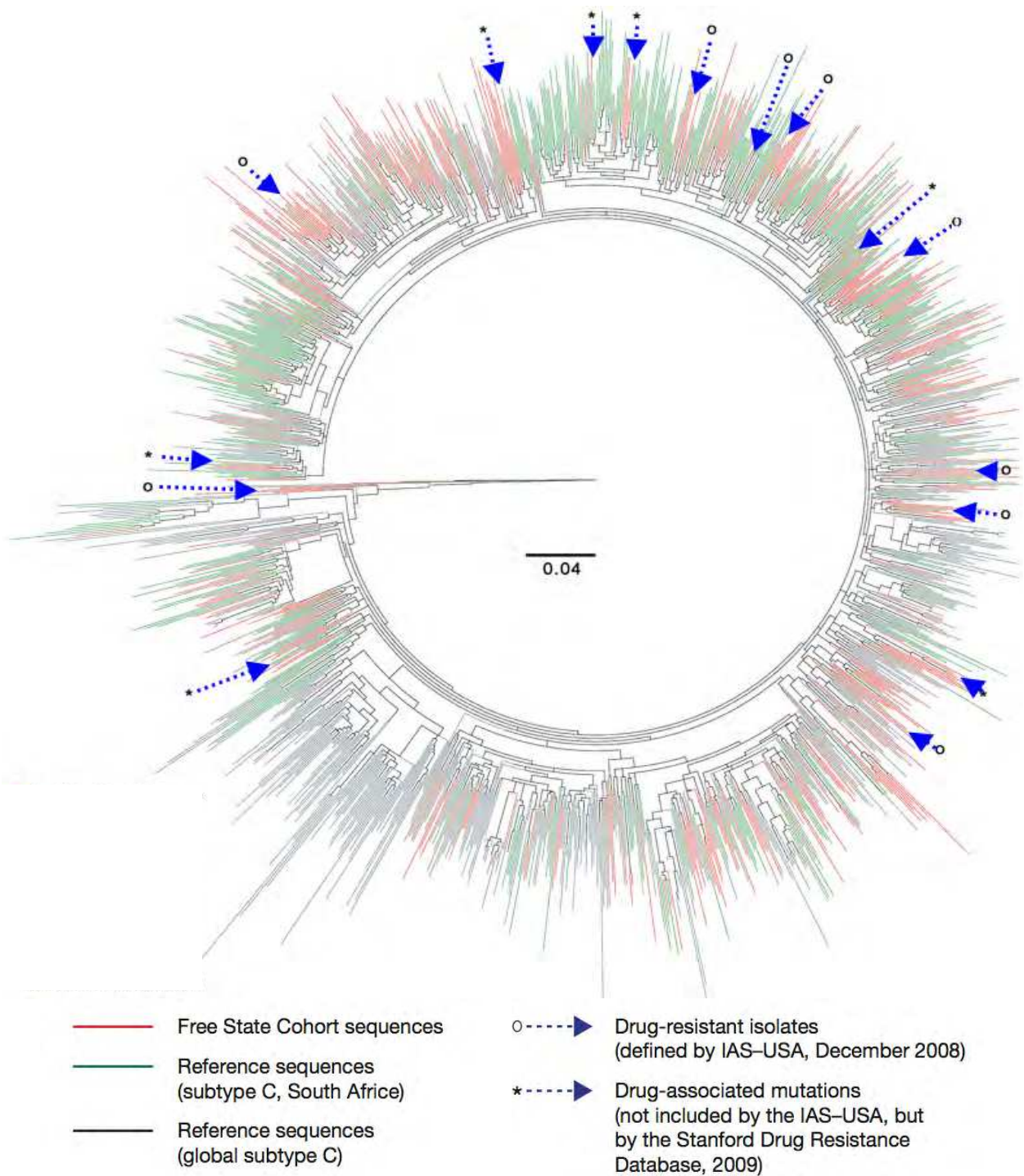
The 884 patients were stratified according to CD4 T cell count, and viral sequences were analysed from 425 patients to form the low (<100 cells/ μ l; n=195), intermediate (200–349 cells/ μ l; n=120) and high (>500 cells/ μ l; n=110) CD4 T cell counts stratification groups. The mean CD4 T cell counts for all patients within the low, intermediate and high groups were 45.4, 270.0 and 651.8 cells/ μ l, respectively. The mean age of the cohort was 36.1 years (Table 1). Complete *protease* gene sequences (99 amino acids) and amino acids 1–530 of the *RT* gene were successfully amplified from 390 and 397 patients, respectively. The REGA subtyping tool revealed all isolates to be subtype C. All sequences were combined with reference sequences from the BioAfrica database and their phylogeny analysed using maximal likelihood trees (de Oliveira and Cassol 2011, de Oliveira, et al. 2005).

5.2 Molecular epidemiology studies revealed dynamic relationships between the local circulating viral isolates and the Southern Africa strains

The Free State is located in central South Africa and is relatively isolated from other South African centres. The phylogenetic analysis confirmed that Free State province is dominated by HIV-1 subtype C infection, much like the rest of South Africa (Table 1). There were multiple small but distinct clusters within the Free State, which suggests the mixing of HIV-1 strains from across South Africa, with migration into and through the Free State, possibly a result of the mining industry, transport routes and other economic reasons (Figure 2 and 3). However, despite these clusters, viruses with drug-resistance associated mutations are distributed randomly, suggesting that they have evolved recently as a result of individual drug exposure rather than being a transmission cluster.

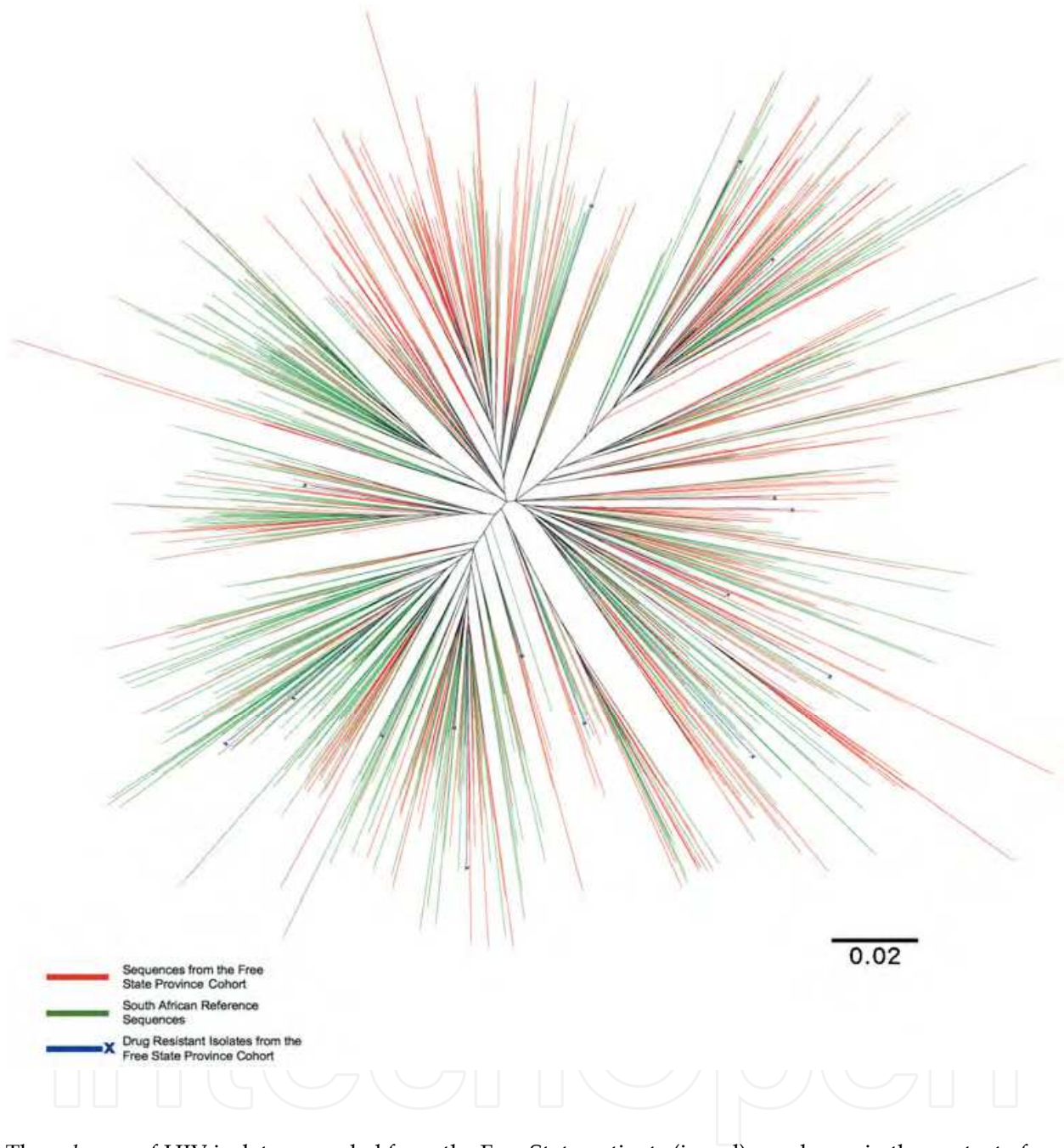
In Figure 2, the maximum likelihood tree shows 390 HIV-1 *pol* sequences from the Free State in the context of local and global HIV-1 subtype C viruses from the BioAfrica database. The Free State strains (n=390) are in red, the South African strains (n=428) are in green, and remaining global subtype C strains (n=551) are in black. In Figure 3, the tree shows the phylogeny of the Free State patients (n=390) in the context of local HIV-1 subtype C virus strains from other provinces within SA (n=428). The Free State strains are highlighted in red, the drug resistant strains are highlighted in blue, whilst the SA reference strains (mainly from Gauteng, Kwa-Zulu Natal and Western Cape Province) are highlighted in green.

The Free State sequences appear to be distributed randomly amongst both Southern African (Figure 2) and South African (Figure 3) reference sequences. Quantitative analysis of clustering showed that 52.5% of sequences occurred in 61 clusters of between 2 and 18 sequences (mean 3.4 patients per cluster: 27 clusters of two, 10 clusters of three, 8 clusters of four, 3 clusters of five, 1 clusters of six, 2 clusters of seven, and 3 individual clusters of eight, nine and eighteen sequences). The analysis also shows that 70.6% of the Free State sequences (in isolation or in clusters) have a South African reference sequence as the nearest neighbour, but there is also clustering of sequences sampled from the Free State compared with other major South African centres (Slatkins and Maddison test, $P < 0.001$). In addition, 96.1% of Free State strains are most closely related to a Southern African sequence and 97.2%



The *pol* gene of HIV-1 isolates sampled from Free State patients (red) are shown in the context of published South African (green) and non-South African (black) HIV-1 subtype C reference sequences. Resistant isolates defined by International AIDS Society (IAS)–USA and other drug-associated mutations defined by the Stanford Drug Resistance Database are shown (blue dotted arrows, ending in “o” and “*”, respectively). This maximal likelihood tree was constructed using the general time reversible substitution model with optimised proportions of invariable sites and gamma distribution (GTR+G+I) using PAUP* (version 4.0 beta) and PhyML (version 2.4.4) software.

Fig. 2. Phylogenetic tree of the HIV-1 molecular epidemiology in the Free State, South Africa in the context of global HIV-1 subtype C *pol* reference sequences



The *pol* gene of HIV isolates sampled from the Free State patients (in red) are shown in the context of published South African HIV-1 Subtype C database (in green). The two HIV population sequences showed remarkable homology, sharing common internal nodes. Only 1 group of 18 Free State isolates clustered with more than 10 sequences. The drug resistant isolates from Free State patients (blue stars) distribute sporadically across the tree. This maximal likelihood tree was constructed using the general time reversible substitution model with optimised proportions of invariable sites and gamma distribution (GTR+G+I) using PAUP* (version 4.0 beta) and PhyML (version 2.4.4) software.

Fig. 3. Phylogenetic tree describing HIV molecular epidemiology of the Free State Province and its drug resistant subjects in the context of South African HIV-1 subtype C reference sequences

of Free State strains are most closely related to an African sequence. Only 2.8% of the Free State sequences are closely related to a non-African sequence.

Sequences that contained any drug-associated polymorphism or a mutation from the IAS-USA surveillance list are indicated and are randomly distributed (Johnson, et al. 2008). The absence of linkage of these variant sequences does not support significant transmission of drug resistance within this cohort (Slatkins and Maddison test, $P=0.85$).

It is also important to note that in contrast to the developed nations, HIV molecular epidemiology remains poorly characterised in the sub-Saharan Africa. In an effort to fight the pandemic in its epicentre, initiatives have begun to enhance the understanding of molecular epidemiology of HIV infection in sub-Saharan African. Consortiums such as Southern African Treatment and Resistance Network (SATuRN) were established as a regional mirror of the Stanford HIV db in sub-Saharan region (SATuRN-Database, et al. 2011). In 2009, only 428 RT sequences were enlisted, with the active contribution from the likes of this cohort ($n=390$), the sample size of SATuRN is expanding rapidly.

5.3 Low prevalence of pre-therapy drug resistance in the Free State

All available pre-therapy *pol* gene sequences ($n=390$ for PR; $n=397$ for RT amino acids 1-530) from the Free State cohort were submitted to the Stanford HIV Resistance Database for identification of polymorphic sites associated with drug resistance. Although all potential resistance mutations were identified, specific attention was paid to the mutations from three drug classes currently being provided as part of the Free State public HAART regimen, namely protease inhibitor (PI), nucleoside reverse transcriptase inhibitor (NRTI), and non-nucleoside reverse transcriptase inhibitor (NNRTI).

According to the Stanford Drug Resistance Database list, the prevalence of non-accessory mutations was 4.1%, comprising 16 patients carrying a total of 19 non-accessory mutations (Table 1, Accessory mutations are defined as “atypical mutations or subtype-associated polymorphisms possibly related to secondary drug resistance” (Stanford University 2011)). The overall prevalence of clinically significant drug resistance mutations (according to the IAS-USA classification) was low (2.3%, Table 1), in concordance with the findings of previous smaller studies from South Africa (Bessong, et al. 2006, Gordon, et al. 2003, Johnson, et al. 2008, Pillay, et al. 2002, Shekelle, et al. 2007). A total of 64 (16.4%) patients sampled had a mutation at any of the drug resistance amino acid sites, including those that have been found to be naturally occurring polymorphisms (Table 1).

The 16 patients with non-accessory mutations – according to the Stanford definition – are detailed in Table 2. Of these, nine had clinically significant mutations according to the IAS-USA list. These were Y181C ($n=2$), K103N ($n=3$), Y188L ($n=1$), V106M ($n=1$), V108I ($n=1$) and K219E ($n=1$) in the RT gene, and M46L ($n=1$) and N88S ($n=1$) in the *protease* gene. The referral centres and local clinics where these patients had been recruited were either visited or contacted; however, in all but five cases the patients had been lost to follow-up. Of the four who had maintained undetectable viral loads on therapy, none had clinically significant IAS-USA defined mutations at baseline (V179D, M46L, A98G and T69N). The one patient identified with a detectable viral load on ARVs (13,000 RNA copies/ml after 5 months) had V179D at baseline, although no sequence data was available from subsequent samples to determine the development of resistance (Table 2). Of the 16 patients with significant resistance, 6 were male. Of the 10 females, the median age was 34.5 years (range 25–56).

Patient identification	Gender	Age, years	CD4+ T-cell count, cells/ μ l	PI major mutation (grade of resistance) ^a	NRTI mutation (grade of resistance) ^a	NNRTI mutation (grade of resistance) ^a	Response to therapy
OX2032	F	35	1	–	–	E138K (potentially low) and Y181C (high)	Lost to follow-up
OX428	M	37	2	–	–	A98G (potentially low)	Lost to follow-up
OX927	F	25	3	–	K219E (low)	Y181C (high)	Lost to follow-up
OX195	M	41	5	–	T69N (potentially low)	–	Lost to follow-up
OX693	M	31	14	–	–	V179D (potentially low)	VL=13,000 copies/ml 5 months after starting therapy
OX1	F	42	23	–	–	K103N (high)	Lost to follow-up
OX613	F	24	28	–	–	V179D (potentially low)	VL<25 copies/ml 28 months after starting 3TC/d4T/EFV
OX1082	M	37	30	–	–	V106M and Y188I (both high)	Lost to follow-up
OX2233	F	28	42	–	–	K103N (high)	Lost to follow-up
OX677	F	31	60	M46L (low)	–	–	VL<25 copies/ml 23 months after starting 3TC/d4T/EFV
OX2011	M	37	63	–	–	A98G (potentially low)	VL<25 copies/ml 33 months after starting 3TC/d4T/EFV
OX5	F	56	64	–	T69N (potentially low)	–	VL<25 copies/ml 36 months after starting 3TC/d4T/EFV
OX1006	M	43	64	–	–	V108I (potentially low)	Lost to follow-up
OX2519	F	32	205	–	–	V179D (potentially low)	Lost to follow-up
OX312	F	38	215	–	–	K103N (high)	Lost to follow-up
OX626	F	39	550	N88S (intermediate)	–	–	Lost to follow-up

The table shows observed pre-therapy drug-associated mutations (class of antiretroviral agent, mutation position and grade of resistance), arranged in ascending order of CD4+ T-cell count and virological response (if known). ^aGrades of resistance are defined using the Stanford Drug Resistance Database. d4T, stavudine; EFV, efavirenz; F, female; M, male; VL, viral load; 3TC, lamivudine.

Table 2. Patients with drug-associated mutations to antiretroviral drugs during pre-therapy assessment

Table 3. Drug-associated mutations in the Free State Cohort

Category	PI		NRTI		NNRTI							
	M46L	N88S	T69N	K219E	A98G	K103N	V106M	V108I	E188K	179D	Y181C	Y188L
Free State Province cohort												
Patients with CD4 ⁺ T-cell count <100 cells/μl (<i>n</i> =192), <i>n</i> ^a	1	0	2	1	2	2	1	1	1	2	2	1
Patients with CD4 ⁺ T-cell count 200–350 cells/μl (<i>n</i> =114), <i>n</i> ^b	0	0	0	0	0	1	0	0	0	1	0	0
Patients with CD4 ⁺ T-cell count >500 cells/μl (<i>n</i> =91), <i>n</i> ^c	0	1	0	0	0	0	0	0	0	0	0	0
Stanford drug resistance database												
Mutation prevalence in drug-naïve subtype B patients, (<i>n</i> =7,404 for PR, <i>n</i> =5,539 for RT), %	0.3	0	0.4	0	0.2	0.3	0	0.6	0.2	2	0	0
Mutation prevalence in drug-naïve subtype C patients (<i>n</i> =2,145 for PR, <i>n</i> =1,979 for RT), %	0.1	0	0.1	0	0.5	0.3	0	0.3	0.2	0.5	0.1	0.1
Other HIV-1 subtypes (drug-naïve) with mutation prevalence ≥0.5% (subtypes A, D, F, G, AE and AG)	Subtype G (0.5%, <i>n</i> =619)	No	Subtype AE (0.5%, <i>n</i> =762)	No	No	No	No	Subtype AG (1.2%, <i>n</i> =1,025)	No	Subtype D (4%, <i>n</i> =320), subtype AE (1.5%, <i>n</i> =762)	No	No
Mutation prevalence in drug-experienced subtype C patients (<i>n</i> =282 for PR, <i>n</i> =1063 for RT), %	2.8	3.2	4.2	7	8.2	28	13	4	1.3	4.5	11	4.1
Genotype–treatment correlation?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Genotype–phenotype correlation?	1.8–8.2	0.1–8.9	NA	NA	NA	22–51	11–356	NA	NA	NA	1.3–148	4.3–400
Genotype–virological correlation?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NA	Yes	Yes	Yes	NA
Mutation grading (Stanford Drug Resistance Database)	Low	Intermediate	Potentially low	Low	Potentially low	High	High	Potentially low	Low	Potentially low	High	High

Table 3 shows the non-accessory drug-associated mutations in the Free State cohort and in reference cohorts (values \geq 0.5% are in bold). The genotype–treatment correlation row shows whether the mutation is associated with therapy. The genotype–phenotype correlation row shows *in vitro* evidence (fold resistance) for associated phenotypic resistance. The genotype–virological correlation row shows whether mutations have an effect on viral load on therapy. All results are summarized from MARVEL and the Stanford Drug Resistance Database. The significance of each resistance mutation is compared with the drug mutation lists of the International AIDS Society–USA and the World Health Organization. *P*-values were calculated using the χ^2 test for trend, comparing the resistance statistics across the three stratified patient populations. The level of significance for clustering of mutations among low CD4⁺ T-cell counts (χ^2 test for trends) was *P*=0.004 for highly active antiretroviral therapy and *P*=0.005 for non-nucleoside reverse transcriptase inhibitors (NNRTIs). Total mutations =16. Total mutations =2. Total mutations =1. HIV-1, HIV type-1; NA, not applicable; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PR, protease; RT, reverse transcriptase.

The non-accessory mutations identified in the cohort are explored in more detail in Table 3. For each mutation, the number in each CD4 T cell counts stratification is shown and compared with the reported prevalence in databases of drug-naïve subtype B and C populations and in drug-experienced subtype C populations. To determine the clinical implications of these identified mutations, the Stanford database MARVEL report for each is summarized and the significance of the mutations in the surveillance lists of the WHO (updated 2009) and IAS-USA (updated December 2008) are compared (Table 3) (Bennett, et al. 2009, Johnson, et al. 2008, Stanford University 2011). For all the mutations, the prevalence in databases of drug-experienced subtype C cohorts was higher than in drug-naïve patients with implications that these mutations might act as markers of drug exposure, even if not conferring clinically relevant resistance.

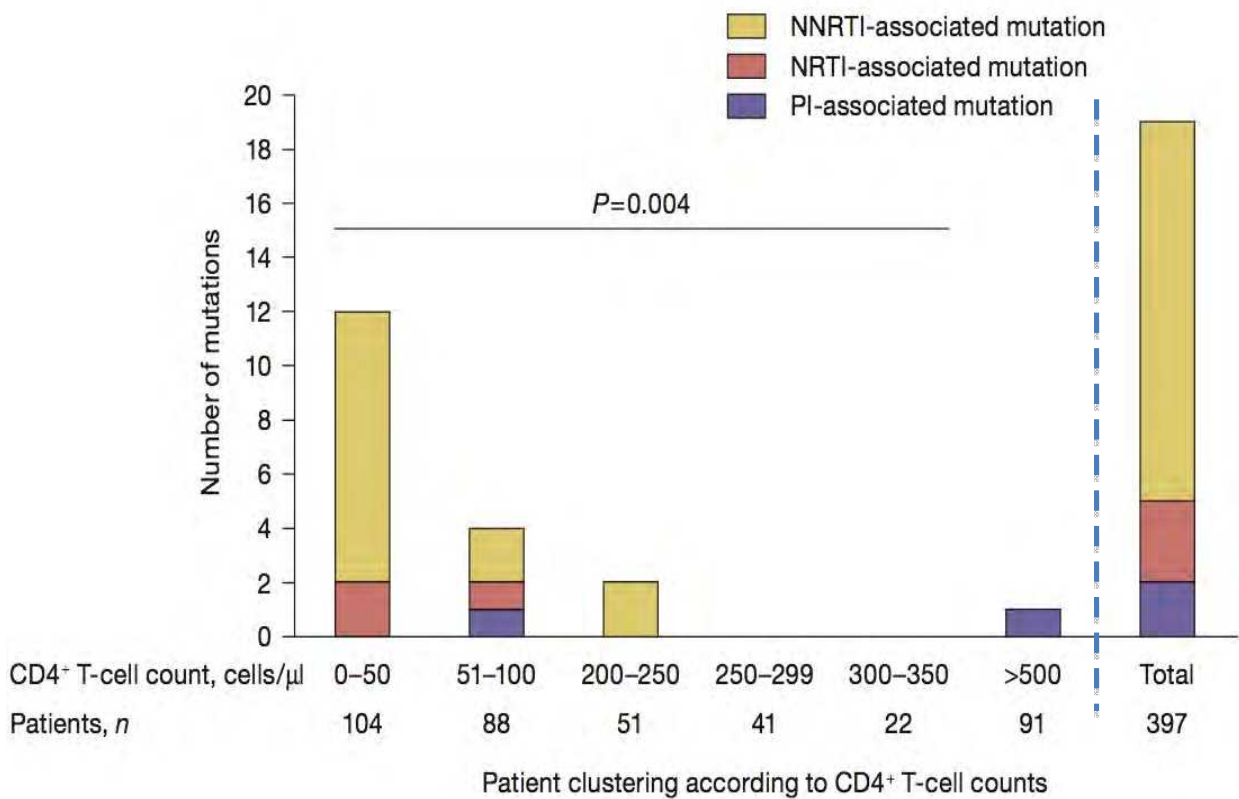
5.4 Enrichment of drug-associated mutations among patients with low CD4 T cell counts

Drug-associated polymorphisms (based on the Stanford database) were concentrated among patients with low CD4 T cell counts – 6.8% of patients with CD4 T cell counts <100 cells/ μ l carried non-accessory mutations compared with 1.8% and 1.2% of patients with intermediate and high CD4 T cell counts, respectively ($P=0.015$; Table 1 and Figure 4). The prevalence of resistance according to the IAS-USA definition was 3.6%, 0.9% and 1.2% for low, intermediate and high CD4 T cell counts groups, respectively (Table 1). Although not statistically significant, there were more accessory mutations among patients with low CD4 T cell counts (19.3%) compared with the intermediate (13.4%) and the high (14.0%) CD4 T cell counts groups. When using the more relaxed Stanford definition, mutations concentrated among patients with low CD4 T cell counts for all mutations (Figure 4; $P=0.004$). Although not statistically significant, when only considering clinically relevant mutations, there was a trend for enrichment of resistance among low CD4 T cell counts for the IAS-USA ($P=0.055$) and the WHO TDRM lists ($P=0.086$; Table 3).

In a supposedly drug-naïve cohort, the enrichment of mutations with low CD4 T cell counts suggests that, rather than being transmitted, these polymorphisms had been selected by drug exposure (Garcia-Diaz, et al. 2008, Jourdain, et al. 2004). Transmitted mutations should be more common in recently infected individuals as they may revert to wild type over time in the absence of therapy (Gandhi, et al. 2003). Chronically infected patients with low CD4 T cell counts would be expected to have relatively less mutations if transmission was the only source of drug resistance. It should also be noted that other evidences have suggested that many of the transmitted resistance can stably persist over prolonged period within the HIV recipient (Brenner, et al. 2002, Novak, et al. 2005, Smith, et al. 2007). However, in the setting of this chronic cohort where majority of the patients are in advanced AIDS and HIV acquisition more likely occurred at a longer time ago, this explanation is unlikely to explain the observed drug-associated polymorphism prevalence given the availability of ARV in the distant past of SA. In this cohort, the patients were counselled by trained local HIV-1 nurses regarding drug history whether through the prevention of PMTCT, ARV programmes in different provinces, countries and private clinics or other non-governmental sources. This cohort does not comply with all the WHO guidelines for the identification of transmitted resistance (i.e. patients with recent infection, aged <25 years and no history of pregnancy (Bennett, et al. 2008, Gilks, et al. 2006, HRDS-Team 2003)), and although transmission of mutations is documented in other cohorts (Gifford, et al. 2007, Gilks, et al. 2006, Pillay 2007,

Richman, et al. 2004), a combination of phylogeny and the association with lower CD4 T cell counts in the data suggested that transmission was unlikely to be a significant cause of resistance in the Free State.

The NNRTI class, in particularly Nevirapine, was associated with significant baseline drug-associated mutations amongst the low CD4 T cell counts patient strata (Jourdain, et al. 2004). The genotypes and phylogeny of the NNRTI resistance strains were heterogenous (Figure 4 and Table 2) and were not derived from any common founder (Figure 2 and 3). Of the patients with low CD4 T cell counts (<100 cells/ μ l), 5.2% possessed non-nucleoside reverse transcriptase inhibitor (NNRTI)-associated mutations compared with 1.8% of patients with intermediate and 0.0% of patients with high CD4 T cell counts ($P=0.005$; Figure 4 and Table 3). The major mutation genotypes observed for NNRTI resistance were K103N (grade 5; 3 isolates), V106M (grade 5; 1 isolate), Y181C (grade 5; 2 isolates), and Y188L (grade 5; 1 isolate). Other NNRTI mutations included K103R (grade 1; 3 isolates), V108I (grade 2; 1 isolate), A98G (grade 2; 2 isolates), V179D (grade 2; 3 isolates), E138K (grade 3; 1 isolate), (Table 2). This association remained significant when restricted to NNRTI mutations on the IAS-USA list ($P=0.031$; Table 3). The distribution of NNRTI mutations within patients with



The plot depicts all pre-therapy patients carrying drug-associated mutations to any of the highly active antiretroviral therapy constituents, including protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), stratified according to CD4 T cell count. The P-value was calculated using the χ^2 test, comparing the mutations across CD4 T cell counts.

Fig. 4. Distribution of drug-associated mutations in pre-therapy patients according to CD4 T cell counts

low CD4 T cell counts was concentrated further among the individuals with lowest CD4 T cell counts (Table 2), who also tended to have higher grade resistant mutations.

In contrast, the prevalence of resistance to non-NNRTI classes of HAART was much lower (Table 2 and 3). Only two major PI resistance mutations in *protease* were observed (M46L (grade 3) and N88S (grade 4)) and two NRTI mutations (T69N (grade 2) and K219E (grade 3)).

Very few individuals were identified with clusters of mutations, indicative of potential multi-class resistance (Table 2). One patient (OX927) had significant resistance to both NRTI (K219E) and NNRTI (Y181C). One patient (OX1082) possessed two significant NNRTI resistance mutations, V106M and Y188L. Another (OX2032) carried E138K and Y181C.

A key route of ARV exposure is the nevirapine-only regimen initially used for PMTCT from 2000 (Department of Health 2003, Jourdain, et al. 2004, Shekelle, et al. 2007). Those patients with major NNRTI mutations were predominantly women of child-bearing age. Nevirapine is an inexpensive and effective agent and a key constituent of HAART regimens in resource-limited settings. In this cohort, nevirapine had the highest prevalence of drug-associated mutations. The drug has a long half-life, a simple mutational pathway and is prone to rapid resistance even with the single doses used in PMTCT (Grossman, et al. 2004, Jourdain, et al. 2004, Shekelle, et al. 2007). Unfortunately, despite contacting or visiting the referring clinics, the demographic details to determine the extent of this effect were not available for this analysis because of loss of follow-up for most of the affected patients.

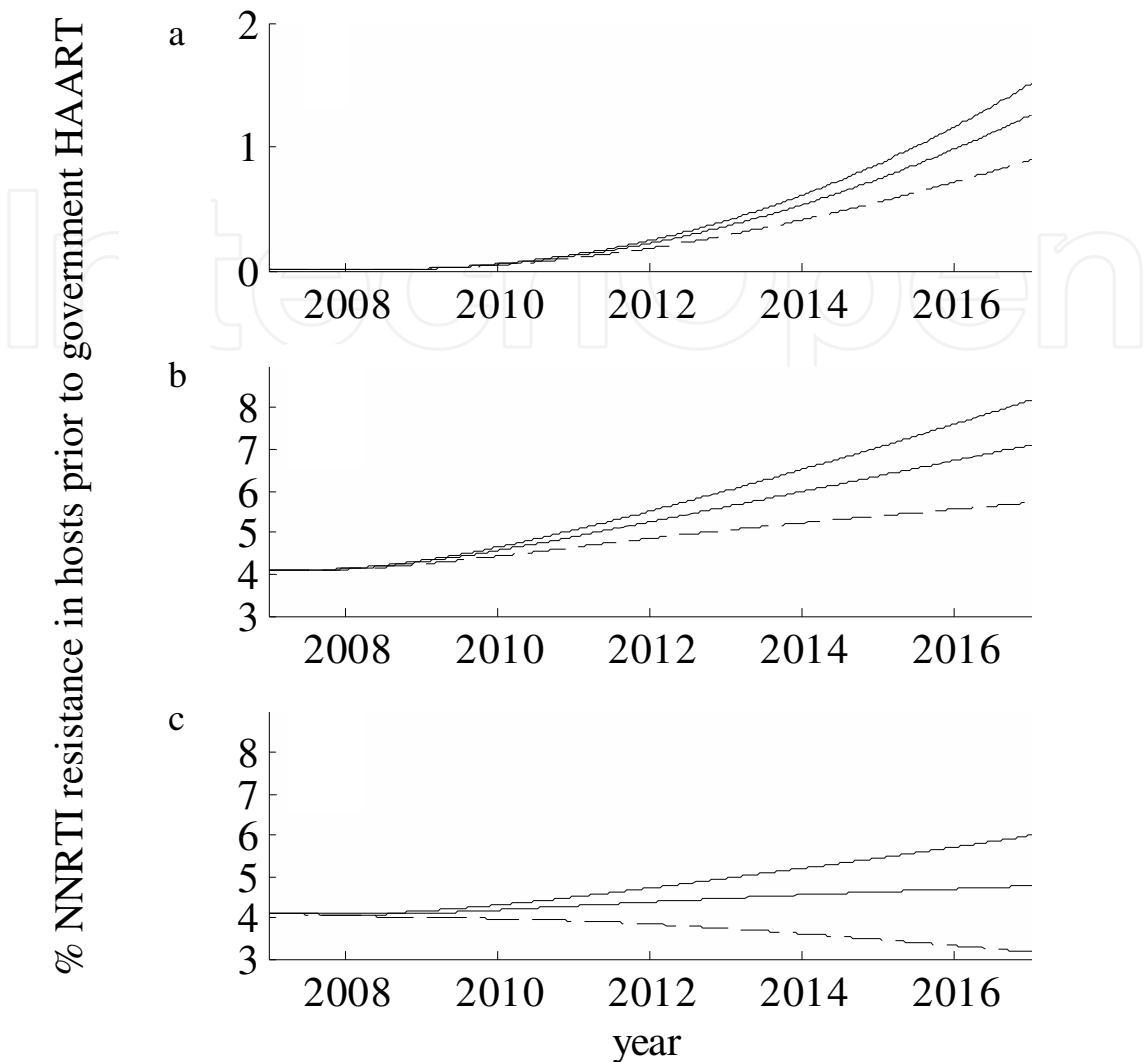
5.5 Mathematical modelling of acquisition of drug resistance

The presence of inducible drug-resistance mutations in a population prior to recruitment to the ARV program indicates either access to antiretroviral therapy from alternative routes or transmission of drug-resistant strains. A mathematical model was developed to address two questions. Firstly, how long would it take for the observed level of inducible resistance mutation prevalence to develop in the absence of non-governmental access to drugs? Secondly, if alternative routes of drug access are maintained at the current rates, what is the likely prevalence of drug resistance in the next ten years?

Regarding the first question on the attributable impact on baseline inducible drug resistance of non-governmental access to ARVs, the model estimates that it should take between 20 and 35 years for a treatment naïve population exposed to NNRTI as part of HAART to achieve the observed level of resistance in the Free State cohort (Figure 5a). This supports the existence of additional sources of NNRTI exposure and that the Free State population is not entirely treatment naïve before the government program.

In answer to the second question, the model predicts that in patients with low CD4 T cell counts the prevalence of NNRTI resistance will increase to 7.1% (confidence interval (CI): 5.7 to 8.2%) over the next 10 years, if the additional sources of NNRTI exposure are maintained (Figure 5b). The resistance prevalence can however be stabilised to 5.2% (CI = 3.6 to 6.2%) over next 10 years, if additional exposure to NNRTI causing resistance can be reduced to 1% (currently 4.2%, Figure 5c). If all additional NNRTI exposure could be restricted, the present resistance prevalence could not sustain itself and would be expected to fall.

Therefore, according to the mathematical model, the prevalence of NNRTI resistance is likely to increase, if additional non-governmental ART exposure is not controlled. The



Panel A: A projection of how resistance would develop in the absence of alternative, non-governmental sources of ARTs, if there was no initial resistance to NNRTI.

Panel B: A projection of how NNRTI resistance will increase over the next 10 years if the percentage of infected hosts acquiring resistance from alternative access to therapy continues at the current level of 4% (estimated using the model).

Panel C: A projection of resistance emergence over the next 10 years, if the percentage of infected hosts acquiring resistance from alternative access to therapy reduces to 1%.

Limited data were available to estimate the rate at which drug resistant virus reverts to drug sensitive virus in untreated hosts (ψ), hence all model predictions were made for three different reversion rates: $\psi = 0.13 \text{ years}^{-1}$ (dashed line), $\psi = 0.05 \text{ years}^{-1}$ (solid line) and $\psi = 0.01 \text{ years}^{-1}$ (dotted line).

Fig. 5. Model predictions of how the prevalence of NNRTI resistance in hosts, prior to enrolment onto governmental ARTs, is expected to develop over time in the Free-State population

implications of this would include reduced drug efficacy and a requirement for clinical drug resistance surveillance, with its inherent financial costs. Newer antiretroviral classes, such as integrase and CCR5 inhibitors are currently unavailable through SA government programs re-enforcing the need to preserve the NNRTI class (Department of Health 2003). In

December 2007, South Africa revised its PMTCT regimen to a combination of zidovudine and nevirapine. Although this measure should improve PMTCT efficacy and reduce the emergence of NNRTI resistance, the logistics of introducing this change might delay its implementation in all areas of South Africa and a proportion of mothers might still receive nevirapine monotherapy.

Nonetheless, PMTCT cannot be responsible for all the observed resistance in the cohort – 6 of the 16 patients were men and the age of the females ranged from 25 to 56 years. These patients predominantly had lower grade mutations, suggestive of exposure but not clinical resistance. It is possible that some individuals had previously received treatment in other clinics, for example, contract workers travelling between provinces. In addition, patients with lower CD4 T cell counts are more likely to be symptomatic, to use drugs prescribed to family or friends and might have previously sought medication through other non-government sources (Novak, et al. 2005, Uy, et al. Jul 2007). At the time of sampling, drugs such as nevirapine, lamivudine, stavudine and didanosine were also available through private practitioners in the Free State, although the duration for which drugs would be prescribed is dependent on the patient's ability to pay (Dahab, et al. 2008, Garcia-Diaz, et al. 2008).

6. Conclusion

In summary, this chapter has reviewed the molecular epidemiology and the practical implications for HIV drug resistance testing in sub-Saharan Africa. The example study of a large cohort from South Africa has revealed new molecular epidemiological and drug resistance surveillance data. The prevalence of drug-associated mutations among patients is reassuringly low, but the association with low CD4 T cell counts is previously undescribed and warrants close monitoring of the virological response when these patients start therapy. In particular, with increasing access to antiretrovirals and growing evidence for the role of treatment as prevention, continued robust resistance surveillance mechanisms will be required for the foreseeable future.

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It can be said that now is the best time for everyone infected to become aware of their own HIV status. The state of the art in HIV management progressively reveals that antiretroviral treatment can prevent transmission, as well as chronic damage in the human body, if started early. Unfortunately, antiretrovirals are not widely available in many places, especially in developing countries. In these parts of the world, diagnosis of HIV infection must be kept in the agenda as a priority, in order to understand specific details of local epidemics and as an effort to interrupt the chain of HIV transmission.

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